AD_____

Award Number: W81XWH-06-1-0414

TITLE: Dietary Influences on Alpha-Methylacyl-CoA Racemase (AMACR) Expression in the Prostate

PRINCIPAL INVESTIGATOR: Dr. Margaret Wright

CONTRACTING ORGANIZATION: University of Illinois
Chicago, IL 60612

REPORT DATE: April 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-04-2011	Final	1 APR 2006 - 31 MAR 2011

4. TITLE AND SUBTITLE

Dietary Influences on Alpha-Methylacyl-CoA Racemase (AMACR) Expression in the Prostate

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-06-1-0414

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Margaret Wright

E-Mail: mewright@uic.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Illinois
Chicago, IL 60612

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Alpha Methyl Acyl CoA Racemase (AMACR), a peroxisomal and mitochondrial enzyme, is up regulated in the majority of prostate cancers (PCa). This enzyme is involved in the breakdown of phytanic acid, which is derived primarily through ingestion of dairy and red meat products. This research project focused on examining the relationships between AMACR expression in the prostate and phytanic acid levels in the blood and prostate. Patient recruitment has been completed, with dietary, clinical, and lifestyle data, as well as biological specimens, obtained from approximately 80 patients with Pca. All dietary data has been processed and serum phytanic acid measurements are complete. Quantification of phytanic acid levels and AMACR gene/protein expression in prostate tissue is underway and results are expected shortly. Preliminary analyses suggest that lifestyle behaviors (including diet) influence blood levels of phytanic acid. Ongoing analyses will determine inter-relationships between diet, serum / tissue phytanic acid levels, and AMACR expression in the prosrate.

15. SUBJECT TERMS

AMACR, Prostate Cancer, Dairy, Red meat, Phytanic acid, Pristanic acid

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

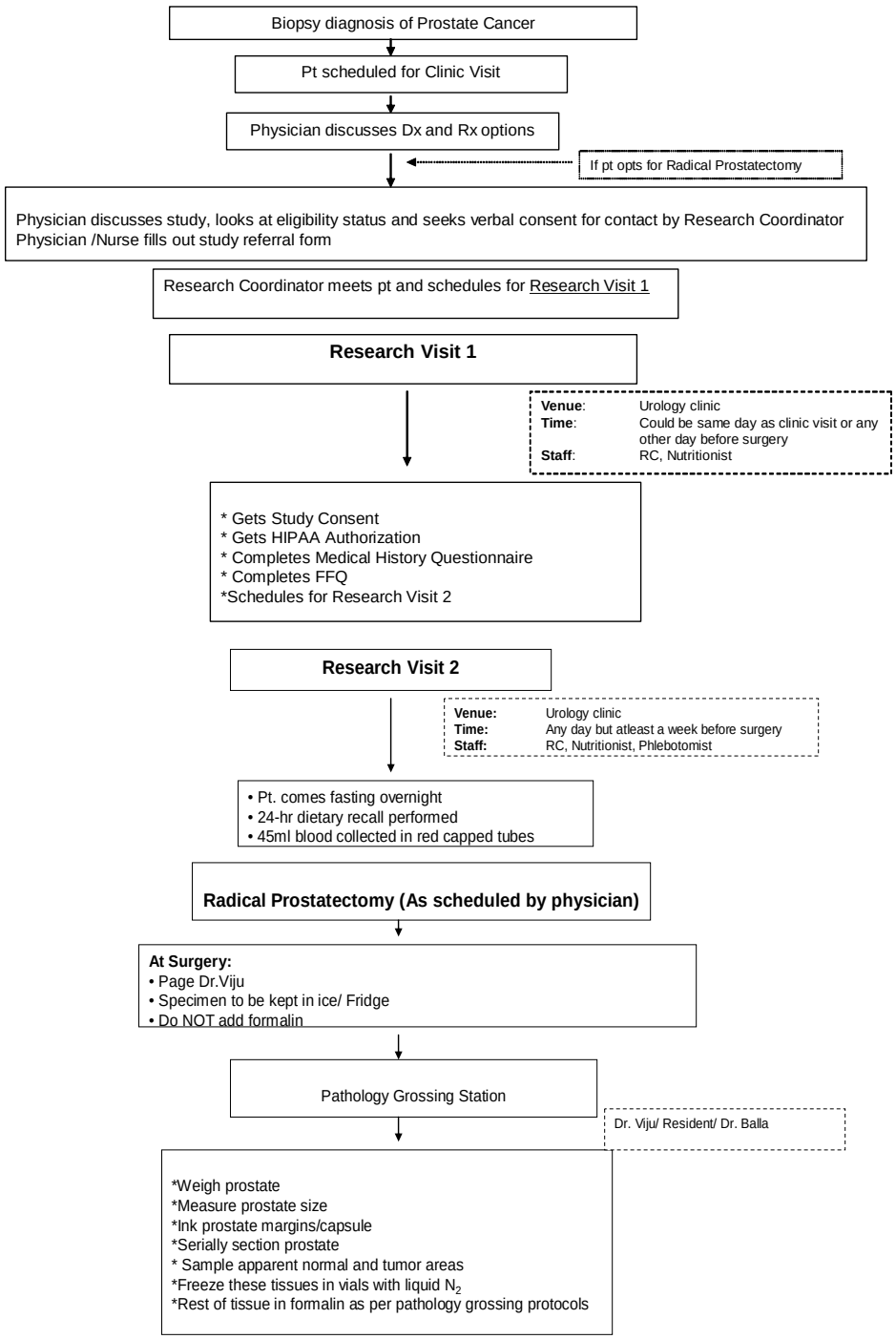
Introduction

Alpha Methyl Acyl coA Racemase (AMACR), a peroxisomal and mitochondrial enzyme, is known to be upregulated in the majority of prostate cancers at the protein and mRNA transcript level. This enzyme is involved in the breakdown of phytanic and pristanic acids - branched chain fatty acids that are derived primarily through the ingestion of dairy and red meat products and cannot be produced de-novo by humans. Although many epidemiologic studies have shown an association between high dairy product / red meat consumption and prostate cancer risk, no study had explored whether consumption of these foods or phytanic acid levels in blood or target tissues are linked to AMACR expression in the prostate. This research project focused on examining the relationships between AMACR expression in the normal prostate gland and phytanic / pristanic acid levels in both blood and prostatic tissue. We postulated that men with higher intake of red meat and dairy products would have elevated phytanic / pristanic acid levels in their blood and prostates, and that this would correspond with higher AMACR expression levels in normal prostate tissues. Forty men with prostate cancer completed the study at the University of Illinois at Chicago (UIC) and the Jesse Brown Veterans Administration Medical Center (JBVAMC). In addition, patient data and biological specimens were obtained from an additional 40 prostate cancer patients at the Henry Ford Health System (HFHS) in Detroit, bringing the *total sample size to 80 men*. Food frequency questionnaires were used to assess red meat and dairy product intake, and gas chromatography-mass spectrometry was used to measure phytanic / pristanic acids in fasting blood (Chicago patients only) and prostate tissues. Determination of AMACR gene expression in the normal prostate by quantitative polymerase chain reaction (qPCR), as well as AMACR protein levels in normal and cancerous tissues by immunohistochemistry (IHC) or immunofluorescence (IF), will be completed shortly. This study will help us to better understand the mechanisms that underlie the consistently observed associations between dairy product / red meat intakes and prostate cancer risk. It should also help us to better understand the exact role of AMACR in prostate carcinogenesis.

Body

The study completed enrollment at two Chicago-based institutions - UIC hospital and JBVAMC – in December, 2010. All men with prostate cancer undergoing radical prostatectomy for the treatment of localized disease were eligible to participate provided they had not received hormonal ablation or neo-adjuvant chemotherapy. Participating subjects completed dietary, lifestyle, and medical questionnaires and provided a fasting 45 mL blood sample prior to surgery. A diagram that depicts patient enrollment and collection of patient data and biological specimens at these two institutions is provided in **Figure 1**.

Figure 1. Schematic Representation of Subject Recruitment in Chicago



Procurement of data and biological specimens from 40 additional patients at HFHS in Detroit, MI is described below.

Research Activities

Please note that in December 2008, Dr. Viju Ananthanarayanan left UIC and Dr. Margaret Wright took over as PI of the project. This change was approved by the UIC Institutional Review Board and the DOD.

We accomplished the following goals as specified in the statement of work included with the original grant proposal.

Institutional Review Board
Institutional Review Board (IRB) approvals from UIC, JBVAMC, and HSRRB were obtained in 2007 and annually thereafter. The addition of data and clinical samples from HFHS was approved by HFHS in 2009 and UIC and the HSRRB in 2010.

Staff Training
Staff involved in the project met training requirements in human subject's protections (including HIPAA research training), blood processing, and handling of biohazardous material before their involvement in the research. Throughout the course of the research project, we had a dedicated study coordinator who was fully trained to recruit participants, collect dietary / medical data, and process biological samples.

Data Management
In 2007, a Microsoft Access® database was developed to record participant information. This database is located on a secure server and access to this database is password protected. The database contains all patient information (including clinical, dietary, and lifestyle data), their surgery dates, and whether prostate tissue and blood samples were obtained. In addition, once a potential participant declined to participate his identifying information was automatically deleted from the database through the use of a macro coded within the database. All patient data has been entered *twice* as a data entry quality control method; discrepancies have been identified and corrected.

Enrollment of Participants

Table 1: AMACR Study Recruitment in Chicago (UIC+JBVAMC)

	Number
Potential Participants	90
Subjects who consented	42
Subjects who withdrew from the study	2
Subjects in the study	**40**
• Completed questionnaires and provided all biological samples	31
• Completed questionnaires and / or provided blood and / or paraffin-embedded tissue, but NO fresh-frozen prostate tissue collected	9

Of the 40 participants recruited at UIC+JBVAMC, 28 are African-American, 11 are white non-Hispanic, and 1 is white Hispanic.

Subject accrual was slower than we originally anticipated. At the JBVAMC, this was largely due to an increase in the number of patients taking Finasteride or Zoladex prior to their radical prostatectomy surgery; both were exclusionary medications for this study. At UIC, recruitment was difficult because the urologists had patient appointments at an off-site location. We therefore requested two no-cost extensions so that we could achieve the enrollment numbers originally stated in the grant. Fortunately, a multidisciplinary oncology clinic opened at UIC in 2010; this clinic enables urologists to see all prostate cancer patients on-site at UIC, and study coordinators are able to meet with potential patients on the same day as their urologist appointment. After the opening of this clinic, we were able to approach every patient that qualified for our study and participation in the AMACR study increased.

A collaboration with Dr. Benjamin Rybicki – Senior Scientist in the Department of Biostatistics and Research Epidemiology at HFHS in Detroit, Michigan – was formalized in 2010 in order to increase the number of AMACR study subjects. Dr. Rybicki had previously carried out an NIH-funded case-control study ("Gene-Environment Interaction in Prostate Cancer" (GECAP)) in which dietary and clinical data, fresh frozen prostate tissue, and formalin-fixed, paraffin embedded (FFPE) tissues were collected from men diagnosed with prostate cancer who underwent radical prostatectomy surgery for the treatment of localized disease. He agreed to provide us with patient data and biorepository specimens from 40 subjects that had participated in his study. Note that fasting blood samples were <u>not</u> collected in Dr. Rybicki's study.

Table 2: Final AMACR Study Enrollment, All Sites (UIC+JBVAMC+HFHS)

	Number
UIC + JBVAMC	40
HFHS (no active recruitment: de-identified data + biological samples only)	40
Total	**80**

Of the 80 participants, 45 are African-American, 34 are white non-Hispanic, and 1 is white Hispanic.

Laboratory Assays

<u>Blood Processing</u>

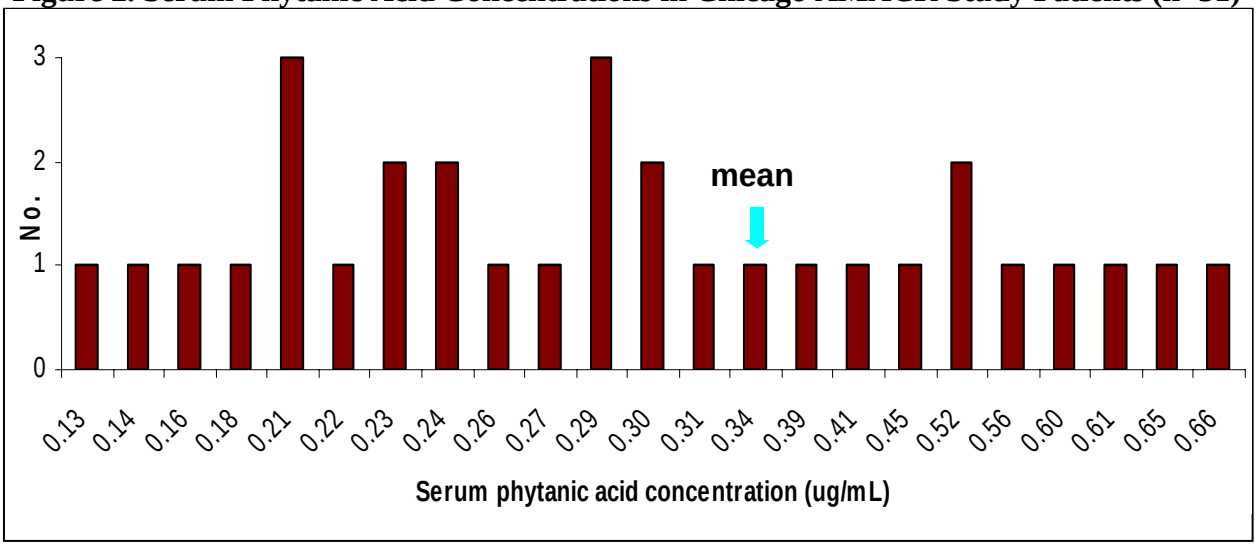
A protocol for processing blood samples was developed and is described in detail in the Appendix of the 2007 Annual Progress report. The protocol enabled us to separate blood components and store them for future use. In addition, it enabled us to cryopreserve white blood cells from many of our patients. We have approximately 271 aliquots of serum, 152 aliquots of plasma, 196 aliquots of red blood cells, and 215 aliquots of white blood cells from our Chicago-based participants. This is an extremely valuable resource for the AMACR study as well as for future studies.

<u>Measurement of Phytanic/Pristanic Acids</u>

Phytanic and pristanic acids are branched chain fatty acids and known substrates for AMACR. These fatty acids were originally going to be measured in the laboratory of Dr. Richard Van Breemen at UIC. Unfortunately, this lab did not demonstrate adequate assay reproducibility and

in 2009 we decided to switch to a different group. Phytanic and pristanic acids have been successfully measured in fasting blood samples from all UIC + JBVAMC subjects by the Peroxisomal Diseases Laboratory at the Kennedy Krieger Institute in Baltimore, MD. This group (co-directed by Dr. Ann Moser) specializes in the analysis of total lipid fatty acids, including very long chain, essential and branched chain fatty acids, by isotope dilution gas chromatography-mass spectrometry (GC-MS). The average intra- and inter-batch coefficients of variation were 1.3% and 12.7%, respectively. The mean serum phytanic acid concentration among UIC+JBVAMC patients was 0.34 μg/mL, with a 5-fold difference in phytanic acid values noted between subjects at the extremes of the distribution (**Figure 2**).

Figure 2. Serum Phytanic Acid Concentrations in Chicago AMACR Study Patients (n=31)



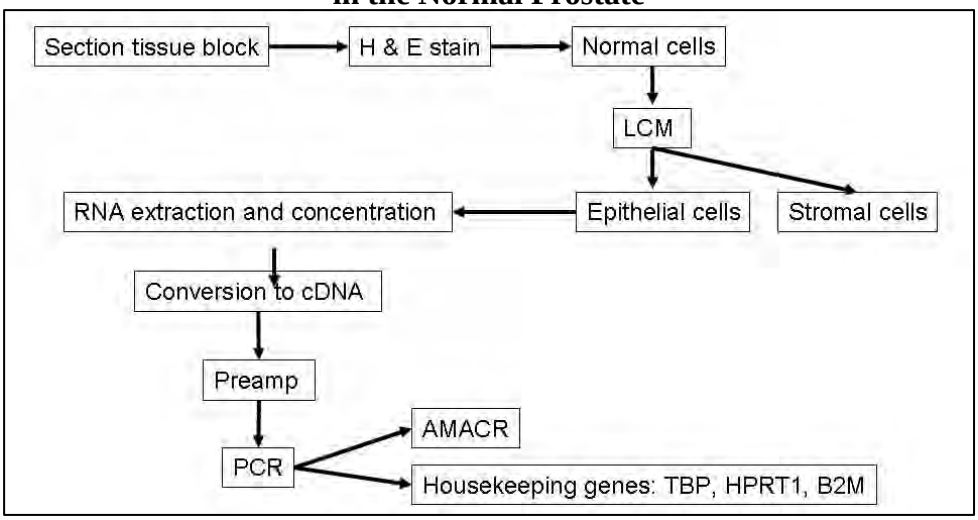
Note that we could not measure circulating phytanic acid levels in HFHS patients because they did not collect fasting blood samples.

Regarding fatty acid measurements in the normal prostate gland, Dr. Moser's group needed to optimize the phytanic acid assay using very small amounts of tissue (<100mg) since HFHS samples were smaller than originally anticipated. Furthermore, the lipid content and percentage of branched chain fatty acids is generally low in prostate tissue. Dr. Moser's group found that the optimal procedure involves lipid extraction and purification of the glycerol lipid fatty acid esters prior to GC-MS, and also determined that a minimum of 75 mg of prostatic tissue was required for reproducible measurements. The tissue phytanic / pristanic acid assays are currently underway and will be completed shortly.

AMACR mRNA Expression

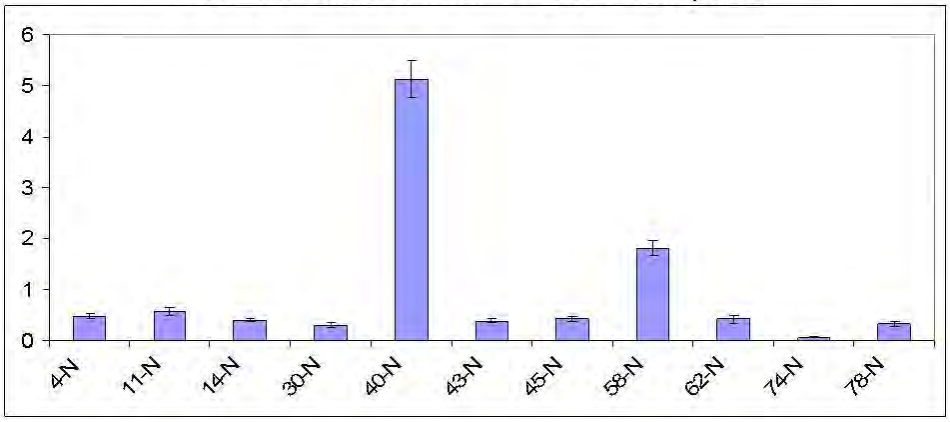
As specified in our aims, we are also evaluating AMACR mRNA expression in fresh prostate tissue sampled at the time of grossing. AMACR mRNA is quantified in normal epithelial areas of the prostate gland. For each patient, this is accomplished by sectioning the fresh-frozen tissue, having a pathologist evaluate a Hematoxylin and Eosin (H & E) stained slide to determine the location of normal cells, and then performing Laser Capture Microdissection (LCM) in order to isolate epithelial cells from stromal cells (**Figure 3**). Once normal epithelial cells are obtained, RNA is extracted with the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion Inc., Austin,

Figure 3. Protocol for Determination of AMACR Gene Expression in the Normal Prostate



TX, USA) according to the manufacturer's protocol with the following modifications: protease digestion at 50º C for 4 hours and DNase digestion at 37º C for 1 hour. RNA quality and quantity is measured on the NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and RNA is stored in RNA Storage Solution at -80º C. 10 ng of total RNA is the input for the RT reaction. RNA is isolated and mRNA expression analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) [1] as using RETROscript RT® (Ambion, Austin, TX, USA) and SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Oligo forward and reverse primers have been designed and optimized for AMACR (5'- agctggccacgatatcaact -3', 5'-ggcatacggattctcaccac-3'). Expression is normalized to three housekeeping genes; TATA-box binding protein (TBP), hypoxanthine phosphoribosyl transferase 1 (HPRT1), and beta-2 microglobulin (B2M). qPCR is run and analyzed on the Applied Biosystems 7900HT Real-Time PCR System.

Figure 4: AMACR mRNA Expression Levels in Normal Prostate Tissues from 11 UIC Participants



LCM, RNA extraction / concentration, and conversion to cDNA has been completed for all UIC + JBVAMC patients. In addition, pre-amplification and PCR has been completed for a subset of these patients; as shown in **Figure 4**, there is wide variability in AMACR gene expression in normal prostate tissues between subjects. Pre-amplification and PCR for the remaining Chicago patients is underway, and LCM / RNA extraction / cDNA conversion / pre-amplification and PCR for HFHS samples is also expected to be completed shortly.

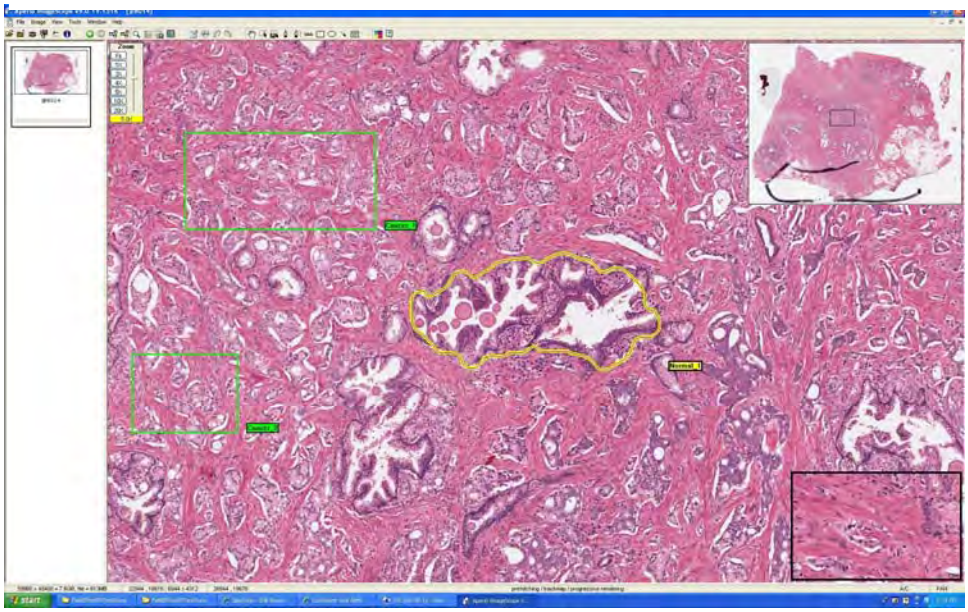
<u>AMACR Protein Expression</u>

As mentioned in the aims, we intend to study AMACR expression at the protein level using immunohistochemistry (IHC) and/or immunofluorescence (IF). Quantification of AMACR can

be done manually or using state of the art image analysis systems. As manual scores are semi-quantitative in nature, use of image analysis systems for quantification provides continuous and reproducible measurements especially at lower expression levels. We have developed two novel approaches: one using standard brightfield digital microscopy (ScanScope®, Aperio Corp.) and one using brightfield or fluorescence multispectral imaging (Vectra®, CRi, Inc.) for automated scoring of tissue images.

Scanscope® is a digital bright field microscope with automatic slide scanning properties. The Scanscope® scans glass slides into digital slide images. These images can then be viewed, browsed and scored over the web. There are inbuilt scoring algorithms that can be used to quantify expression of nuclear and cytoplasmic markers. In addition, we added Spectrum™ Plus to the ScanScope hardware. Spectrum Plus is comprehensive, web-based digital pathology information management software developed for digital slide viewing and conferencing, workflow management, data archival, and image analysis. This system greatly facilitates digital microscopic image review and analysis by providing whole slide digital images and enabling the user to view multiple images simultaneously. In addition, image analysis throughput is greatly increased owing to batch image analysis capabilities. A representative screen shot of Aperio viewing software and Spectrum™ Plus is shown below (**Figure 5**).

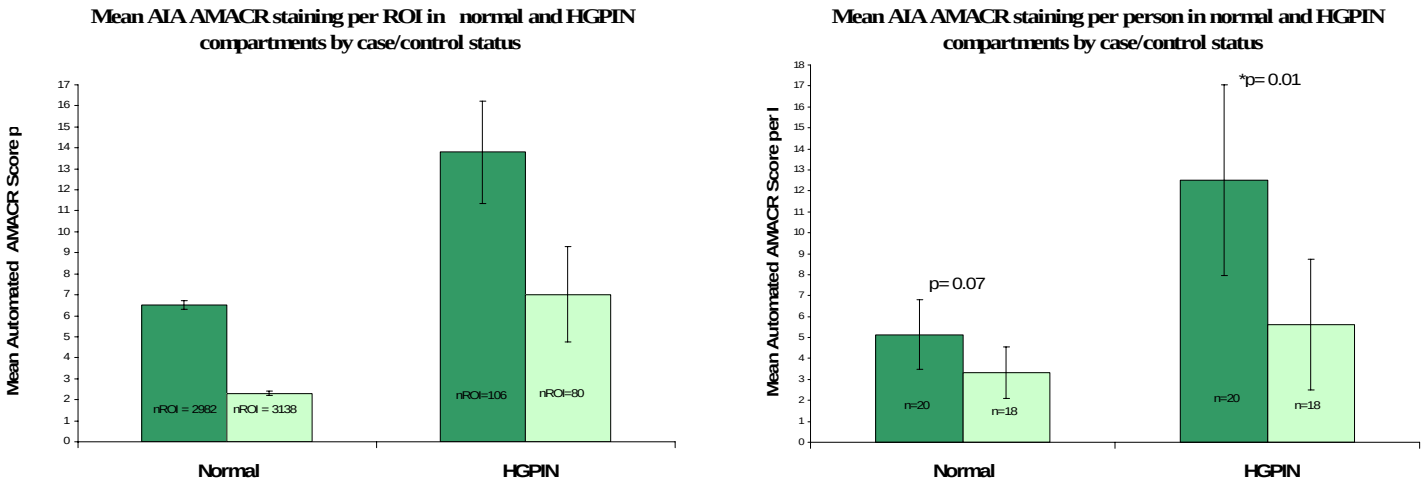
Figure 5: Annotation of Histologic Compartments on a web slide within Spectrum™ Plus and ImageScope®



Previously we showed that AMACR expression in normal glands from patients subsequently diagnosed with prostate cancer was higher than those who did not. No difference was observed for AMACR expression in HGPIN glands [2]. To validate scoring algorithms, we scanned the same biopsy set using the ScanScope CS and digital web slides were created. Regions of interests (ROI) were drawn within benign and HGPIN compartments and scored using 'Positive Pixel Count™' and 'Co-Localization' algorithms of the Aperio software. Separate scores for benign and HGPIN glands were computed as the product of percent area positive and mean intensity of staining. We found that with automated image analysis, normal as well as HGPIN

glands showed higher AMACR expression in biopsies with a subsequent diagnosis of cancer (**Figure 6**). This work was presented at the 2007 Frontiers in Cancer Prevention Research Meeting (see the Appendix of the 2008 Annual Report).

Figure 6: AMACR quantification in Prostatic Biopsies with HGPIN using Automated Image Analysis (AIA)



Vectra® is a new digital microscopy system for whole-slide analysis of either brightfield or fluorescent images. It incorporates two important technologies that make it more powerful and efficient than other current systems for quantitative digital analysis of biomarkers in tissue sections. The first is multispectral analysis: colors are separated by recording the wavelength signature across the color spectrum for each pixel in the image. This permits simple elimination of autofluorescence from images and also allows for clear separation of several color targets contained within a single image. The second important technology is "intelligent" software for pattern recognition (a.k.a. "machine learning"). Investigators can train Vectra to recognize only the tissue compartments or regions of interest while ignoring others. For example, after pre-processing a slide with these algorithms, a user can obtain data on targets that lie only within epithelial (e.g., within a "tumor mask") as opposed to stromal areas.

All FFPE blocks and H & E slides have been obtained for patients enrolled at JBVAMC; we have requested blocks and slides for patients enrolled at UIC and expect to receive them shortly. H&E slides will be mapped in detail for cancer and normal areas and will be used to select appropriate FFPE blocks for AMACR protein quantification via IHC or IF. Whole sections will be cut and immunostained for AMACR using a monoclonal antibody as per standardized protocols at the Research Pathology Core Laboratory at UIC. Suitable positive and negative controls will be run with each staining batch and precautions to minimize inter-batch variability will be undertaken. Note that HFHS specimens were embedded into a tissue microarray as part of the original study at that institution, and we have requested sections for AMACR protein quantification in normal and tumor tissue.

Primary Cell Cultures

We developed protocols for generating primary cell cultures from normal and tumor areas from prostatectomy samples of our study subjects. A small portion of apparently normal and tumor

regions sampled at the time of grossing were used to develop these cell cultures. These cell cultures are different from cell lines by virtue of their limited doublings. Details about the protocol for developing primary cultures are available in the Appendix of the 2008 Annual Progress Report. Primary cell cultures are valuable *in vitro* model systems to study cancer prevention and treatment strategies. We obtained IRB approval to develop these cell culture systems and have primary cell cultures growing for 8 subjects.

Key Research Accomplishments

A summary of key research accomplishments over the course of the award period is listed below:

1) Establishment of a secure database for subject data and biospecimen tracking
    - Double data entry as a quality control measure

2) Formalized collaboration with Dr. Ben Rybicki at the Henry Ford Health System in Detroit

3) Recruitment of subjects completed
    - 40 patients from UIC+JBVAMC (Chicago) and 40 from HFHS (Detroit) = 80 total

4) All dietary questionnaires processed and nutrient data received from NutritionQuest, Inc.

5) Completion of serum phytanic / pristanic acid assays

6) Optimization of phytanic / pristanic acid assays in normal prostate tissue completed

7) Completion of LCM and RNA extraction from normal prostatic epithelial cells for all Chicago patients
    - AMACR mRNA expression completed via rt-PCR in a subset of these patients

8) Image analysis development for quantifying AMACR protein expression in tissue

9) Digitized web slide database established

10) Paraffin-embedded tissue and corresponding H&E slides obtained for all JBVAMC patients; requested for UIC + HFHS patients

11) Analysis of demographic, lifestyle, and clinical determinants of blood phytanic acid levels in Chicago study participants

Preliminary Findings, Ongoing Analyses, and Planned Manuscripts / Presentations

<u>Preliminary Findings</u>
We examined whether demographic, lifestyle, and clinical characteristics are important determinants of circulating phytanic acid levels (see poster in Appendix) and presented our

findings at the 2011 DOD Impact Conference in Orlando, FL. We found that vitamin supplement use and consumption of high amounts of fat, especially saturated fat, were correlated with increased blood levels of phytanic acid, whereas fruit and vegetable intake was inversely correlated with concentrations of this fatty acid. Alcohol use and smoking were also suggestively linked with lower phytanic acid concentrations.

Ongoing Analyses

1) Continued exploration of correlations between serum phytanic acid levels and individual dairy products and ruminant meats (milk, cheese, yogurt, cream, butter, beef, lamb)
2) Evaluation of associations between dietary intakes of red meat / dairy products and phytanic acid concentrations in normal prostate tissue
3) Evaluation of associations between dietary intakes of red meat / dairy products and AMACR gene and protein expression in prostatic tissue
4) Evaluation of associations between serum / prostatic phytanic acid concentrations and AMACR gene and protein expression in prostatic tissue

Planned Manuscripts

1. Dietary, serum, and prostatic phytanic acid in relation to AMACR gene expression in the normal prostate
2. Dietary, serum, and prostatic phytanic acid in relation to AMACR protein expression in the normal and cancerous prostate

Planned Abstract Submissions / Poster Presentations

1. American Association for Cancer Research 103rd Annual Meeting, 2012. "Dietary, serum, and prostatic phytanic acid in relation to AMACR gene expression in the normal prostate"
2. American Association for Cancer Research Advances in Prostate Cancer Research Meeting, 2013. "Dietary, serum, and prostatic phytanic acid in relation to AMACR protein expression in the normal and cancerous prostate"

Reportable Outcomes

Poster Presentations

Wright ME, Ananthanarayanan V, Enk-Reuter E, Deaton R, Ouedraogo B, Moser A, Gann PH. Determinants of Serum Phytanic Acid Concentrations in Men with Localized Prostate Cancer. DOD Impact Conference, 2011. (See Appendix for poster)

Wright ME, Bowen P, Ananthanarayanan V, Virtamo J, Albanes D, Gann PH. A Prospective Study of Phytanic Acid Intake from Red Meat and Dairy Products in Relation to Prostate Cancer Risk. AACR International Conference on Frontiers in Cancer Prevention Research, 2008. (See 2009 Annual Report)
Although the results presented in this poster were not based on samples procured during the DOD study, the findings are directly relevant to this project - they indicate that higher phytanic acid intake is associated with a significant increase in the risk of advanced prostate cancer in a prospective epidemiologic study.

Ananthanarayanan V, Deaton RJ, Poon R, Gann PH. Validation of Automated Image Analysis Methods for Evaluation of AMACR Expression in Prostate Biopsies with High Grade Prostatic Intraepithelial Neoplasia. AACR International Conference on Frontiers in Cancer Prevention Research, 2007. (See 2008 Annual Report)

Oral Presentations

2010	Division of Epidemiology and Biostatistics Seminar, University of Illinois at Chicago School of Public Health (Wright)
2009	Department of Kinesiology and Nutrition Seminar, University of Illinois at Chicago (Wright)
2008	Translational Research and Cancer Prevention and Control Minisymposium, Northwestern University, Chicago, IL (Wright)
2008	Quantitative Imaging Cytometry Symposium, Cold Spring Harbor Laboratory, NY (Ananthanarayanan)
2008	Prostate Cancer Research Working Group Seminar, University of Illinois at Chicago (Wright / Ananthanarayanan)

Personnel Receiving Pay (not salaries) from the Research Effort

Ann Moser, Kennedy Krieger Research Institute, Baltimore MD. Measured phytanic/pristanic acids in serum and prostate tissue samples.

Conclusions

There is a crucial need to identify the biological pathways through which diet affects prostate carcinogenesis. Results of this study could help define important causal links between suspected dietary risk factors for prostate cancer and clinically relevant biomarkers like AMACR. Earlier studies have shown that increased AMACR expression in the normal prostate could be a characteristic of high-risk tissue. If our results confirm a link between diet and AMACR expression, the following inferences will be strengthened: 1) the associations between red meat and dairy intake and prostate cancer risk will be more biologically plausible, leading to the possibility of dietary risk reduction and better identification of high-risk men, and 2) the phytanic/pristanic acid/AMACR pathway will be a more enticing target for discovery of chemopreventive and possibly therapeutic agents. This project could make a number of methodological contributions as well, including the determination as to whether simple food frequency questionnaires or serum samples are capable of predicting tissue levels and whether advanced image analysis techniques provide an important advantage in evaluating pre-malignant changes in tissue.

We successfully enrolled 40 subjects into the study at UIC / JBVAMC. As explained in the Research Activities section, the rate of accrual at these two institutions was slower than

originally anticipated. A collaboration with Dr. Rybicki - a Senior Scientist at HFHS in Detroit – was therefore established in order to increase the number of subjects in our study. Dr. Rybicki has direct access to a biorepository that contains dietary data, fresh-frozen prostate tissue, and FFPE tissue specimens obtained from men undergoing radical prostatectomy for the treatment of localized disease, and he provided us with existing dietary and clinical data, as well as biological samples, from 40 of these men. The final sample size achieved was 80 patients, which is consistent with the recruitment goal stated in the grant. Even though we achieved our target sample size, slow recruitment in Chicago delayed timely completion of several of the biomarker assays, as well as data analysis and manuscript preparation. Two no-cost extension periods were granted, during which time we completed patient enrollment, had phytanic/pristanic acid concentrations measured in fasting serum samples from all Chicago patients, had all dietary questionnaires scanned and electronically processed, extracted RNA from normal epithelial prostatic tissues from all Chicago patients, and began data analyses. At the present time, tissue phytanic acid assays are underway and rt-PCR assays for AMACR gene expression are being completed. FFPE blocks and corresponding H&E slides have been procured for most Chicago patients, and we are awaiting the arrival of TMA blocks from HFHS. Once these blocks and slides are received, we will quantify AMACR protein levels using IHC and/or IF. Data analysis is ongoing and we anticipate submission of two abstracts in the fall of 2011 for presentation at national conferences, as well as submission of two manuscripts that detail findings from the DOD-AMACR Study.

References

1. Nonn L, Vaishinav A, Gallagher L, Gann PH: mRNA and micro-RNA expression analysis in laser-capture microdissected prostate biopsies: valuable tool for risk assessment and prevention trials. Exp Mol Pathol 2010, 88:45-51.

2. Ananthanarayanan V, Deaton RJ, Yang XJ, Pins MR, Gann PH: Alpha-methylacyl-CoA racemase (AMACR) expression in normal prostatic glands and high-grade prostatic intraepithelial neoplasia (HGPIN): association with diagnosis of prostate cancer. *Prostate* 2005, 63:341-346.

Appendix

- **Poster:** *"Determinants of Serum Phytanic Acid Concentrations in Men with Localized Prostate Cancer"*

Determinants of Serum Phytanic Acid Concentrations in Men With Localized Prostate Cancer

Margaret E. Wright[1], Viju Ananthanarayanan[1], Erika Enk-Reuter[1], Ryan Deaton[1], Boubacar Ouedraogo[1], Ann Moser[2], Peter H. Gann[1]

[1]University of Illinois at Chicago, Chicago, IL; [2]Kennedy Krieger Institute, Baltimore, MD

UIC Department of Pathology, University of Illinois at Chicago, College of Medicine

Abstract

Background and Objectives: Phytanic acid, a branched-chain saturated fatty acid found almost exclusively in red meat and dairy products, has been linked to an increased risk of prostate cancer in several studies. Our objective was to evaluate demographic, lifestyle, and clinical factors that may contribute to inter-individual variability in circulating phytanic acid concentrations in men.
Methods: Phytanic acid concentrations were determined by isotope dilution gas chromatography-mass spectrometry in fasting serum samples obtained from 31 men who were scheduled to undergo radical prostatectomy for the treatment of localized prostate cancer. All of these men are participants in the ongoing DOD-funded "Dietary Influences on Alpha-Methylacyl-CoA-Racemase (AMACR) Expression in the Prostate" study. Selected demographic and lifestyle factors were assessed by questionnaire and clinical information was abstracted from medical reports.
Results to Date: Serum phytanic acid concentrations were suggestively associated with use of vitamins and supplements (p=0.08), current alcohol use (p=0.11), and smoking status (p=0.18), with higher concentrations observed in supplement users, nondrinkers, and nonsmokers. Among foods and nutrients, fruit and vegetable intake was inversely correlated (r= -0.57, p=0.009) whereas total (r=0.34, p=0.14) and saturated fats (r=0.45, p=0.04) were positively correlated with blood levels of phytanic acid.
Conclusions: Vitamin supplement use and higher fat intake were associated with higher circulating concentrations of phytanic acid, whereas smoking, drinking, and fruit and vegetable intakes were associated with lower concentrations of this fatty acid.

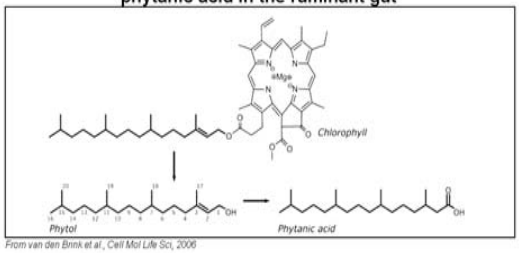
Background

- Higher red meat and dairy product consumption has been linked to elevated risks of prostate cancer in multiple epidemiologic studies.
- These foods are major sources of phytanic acid - a 3 methyl branched chain saturated fatty acid - in Western diets.
- Bacteria in the intestinal tract of ruminant animals degrade chlorophyll into phytol, which is further broken down to phytanic acid (**Figure 1**). Phytanic acid is then deposited in the fat of ruminants.
- Humans obtain phytanic acid almost exclusively from ruminant meat and dairy products as we are unable to release phytol from chlorophyll.
- Alpha-methylacyl-CoA-racemase (AMACR) plays a critical role in the peroxisomal metabolism of phytanic acid.
- AMACR is consistently overexpressed in prostate tumors compared to benign tissue, and is used clinically to resolve ambiguous biopsies.
- High blood levels of phytanic acid have been associated with increased risks of prostate cancer, possibly due to elevations in oxidative stress and / or increased AMACR activity.

Objective

To evaluate demographic, lifestyle, and clinical factors that may contribute to inter-individual variability in circulating phytanic acid concentrations in men.

Figure 1. Conversion of phytol from chlorophyll to phytanic acid in the ruminant gut



From van den Brink et al., Cell Mol Life Sci, 2006

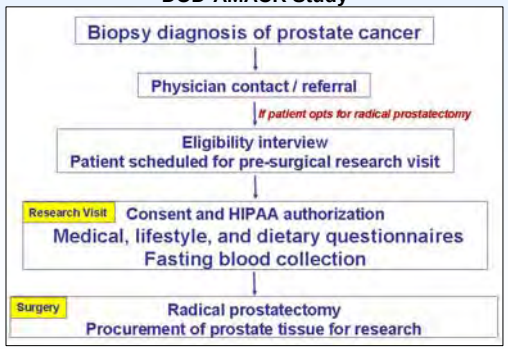


Methods

Study population

- Patients with biopsy confirmed prostate cancer from the University of Illinois Hospital, the Jesse Brown Veterans Affairs Medical Center, and the Henry Ford Health System.
- All men scheduled to undergo radical prostatectomy surgery were eligible to participate, provided that they had not received neo-adjuvant or hormonal ablation therapy beforehand.
- A pre-surgical research visit was completed in order to obtain dietary and lifestyle data, medical history, and a blood sample (**Figure 2**).

Figure 2. Schematic representation of the DOD-AMACR Study



Data and biological sample collection

- Block Brief 2000 dietary questionnaire (**n=24 subjects**)
- Medical history and lifestyle questionnaire
- Fasting 45mL blood sample (**n=31 subjects**)
- Fresh-frozen normal prostate tissue
- Formalin fixed, paraffin embedded tissue

Serum phytanic acid measurements

- Measured in singlicate using isotope dilution gas chromatography-mass spectrometry assay.
- Mean intra- and inter-batch coefficients of variation based on blinded pooled QC samples were 1.3% and 12.7%, respectively.

Statistical analysis

- Log transformation of serum phytanic acid values to approximate a normal distribution.
- T-tests and ANOVA used to examine whether geometric mean concentrations varied according to participant characteristics.
- Partial correlation coefficients used to investigate associations between serum phytanic acid and dietary intake of foods and nutrients.

Results

Table 1: Mean serum phytanic acid concentrations according to demographic, lifestyle, and clinical characteristics in 31 men with prostate cancer

Characteristics	n	Unadjusted geometric mean (95% confidence interval)	*p-value*[1]
Age (years)			0.42
<60	7	0.34 (0.20–0.56)	
60-64	10	0.34 (0.26–0.43)	
≥65	14	0.27(0.20–0.35)	
Race			0.56
Caucasian	11	0.32 (0.24–0.45)	
African American	20	0.29 (0.24–0.36)	
Current alcohol use			**0.11**
No	18	0.34 (0.28–0.42)	
Yes	13	0.26 (0.20–0.34)	
Smoking status			**0.18**
Never	6	0.41 (0.27–0.62)	
Former	9	0.27 (0.18–0.42)	
Current	16	0.29 (0.23–0.35)	
Body mass index (kg/m^2)			0.38
<25	5	0.32 (0.21–0.49)	
25-29.9	12	0.34 (0.24–0.48)	
≥30	14	0.27 (0.21–0.34)	
Vitamin supplement use[2]			**0.08**
No	21	0.28 (0.22–0.34)	
Yes	10	0.37 (0.28–0.51)	
Family history of prostate cancer			0.41
No	20	0.29 (0.23–0.36)	
Yes	10	0.34 (0.25–0.46)	
Gleason grade[3]			0.21
<7	11	0.29 (0.24–0.36)	
7	18	0.33 (0.26–0.43)	
≥8	2	0.18 (0.006–5.63)	

[1] From t-tests for binary variables and ANOVA tests for variables with ≥3 levels
[2] Use of lycopene, selenium, vitamin E, fish oil, or other supplements within the past month
[3] From surgical pathology reports

Table 2: Partial[1] correlations between serum phytanic acid concentrations and dietary variables in 24 men with prostate cancer

	Mean Intake (SD[2])	Correlation coefficient	*p-value*
Food groups (servings/day)			
Dairy products[3]	1.08 (1.25)	-0.21	0.37
Fruits and vegetables	4.08 (2.41)	**-0.57**	**0.009**
Nutrients			
Calories (kcal/day)	2177 (941)	-0.12	0.61
Total fat (grams/day)	95.3 (46.7)	**0.34**	**0.14**
Saturated fat (grams/day)	31.3 (16.9)	**0.45**	**0.04**
Protein (grams/day)	83.2 (40.3)	-0.16	0.50
Carbohydrate (grams/day)	233 (110)	-0.21	0.38
Calcium (mg/day)	678 (459)	-0.26	0.26
% calories from fat	38.6 (8.7)	0.25	0.29
% calories from protein	15.1 (3.3)	-0.09	0.69
% calories from carbohydrate	43.8 (8.7)	-0.17	0.48

[1] Adjusted for vitamin supplement use, current alcohol use, smoking status, and calories
[2] SD = standard deviation
[3] Daily servings of milk, yogurt, and cheese

Conclusions

- Vitamin supplement use and consumption of high amounts of fat, especially saturated fat, are correlated with increased blood levels of phytanic acid in men with early stage prostate cancer.
- In contrast, fruit and vegetable intake is inversely correlated with blood levels of phytanic acid in this group of men.
- Alcohol use and smoking were suggestively linked with lower phytanic acid concentrations, although these findings were of borderline statistical significance.
- Our findings require confirmation in other, larger studies.

Impact

- This is the first study to determine whether demographic, lifestyle, and clinical characteristics are important determinants of circulating phytanic acid levels in men with prostate cancer.
- If confirmed in other studies, our findings indicate that several modifiable behaviors affect serum phytanic acid levels.
- Primary and secondary prevention strategies could be targeted to men who exhibit these behaviors.

Other Ongoing Work

- Continued exploration of correlations between serum phytanic acid levels and individual dairy products and meats (milk, cheese, yogurt, cream, butter, beef, lamb)
- Evaluation of associations between:
  1) dietary intake of red meat / dairy products and phytanic acid concentrations in normal prostate tissue
  2) dietary intake of red meat / dairy products and AMACR expression (RNA and protein) in normal prostate tissue
  3) serum/prostatic phytanic acid concentrations and AMACR expression

Acknowledgement

Supported by a New Investigator Award (***W81XWH-06-1-0414***) from the Department of Defense